



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

|  |           |  |
|--|-----------|--|
| <b>(51) International Patent Classification<sup>6</sup> :</b><br><b>G01N 33/573</b>  | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 97/28449</b><br><b>(43) International Publication Date:</b> 7 August 1997 (07.08.97)   |
| <b>(21) International Application Number:</b> PCT/IE96/00003<br><b>(22) International Filing Date:</b> 2 February 1996 (02.02.96)<br><br><b>(71) Applicant (for all designated States except US):</b> BIOTRIN INTELLECTUAL PROPERTIES LIMITED (IE/IE); 93 The Rise, Mount Merrion, County Dublin (IE).<br><br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> DOYLE, John, Martin (IE/IE); 27 Kill Abbey, Deansgrange, County Dublin (IE). KILTY, Cormac, Gerard (IE/IE); 34 Dundela Park, Sandycove, County Dublin (IE). MANNING, Fiona, Mary (IE/IE); 36 The Close, Oriynn Park, Lusk, County Dublin (IE).<br><br><b>(74) Agent:</b> ANNE RYAN & CO.; 60 Northumberland Road, Ballsbridge, Dublin 4 (IE).  |           | <b>(81) Designated States:</b> AU, BR, CA, CN, CZ, FI, HU, JP, KR, NO, NZ, PL, RU, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).<br><br><b>Published</b><br><i>With international search report.</i> |
| <b>(54) Title:</b> METHOD OF DETERMINING THE HEPATIC STATUS OF AN INDIVIDUAL, INCLUDING A LIVER TRANSPLANT RECIPIENT<br><br><b>(57) Abstract</b><br><br>A method of determining the hepatic status of a subject comprises measuring the level of the pi glutathione S-transferase ( $\pi$ GST) isoform in a sample of a biological fluid from the subject by an immunoassay specific for the $\pi$ GST isoform, comparing the level of $\pi$ GST measured with the normal range of $\pi$ GST in the biological fluid and, when an increase in $\pi$ GST level relative to the normal range is detected, determining the hepatic status of the subject based on the level of $\pi$ GST in the biological fluid. The method has particular application in the case of liver transplantation enabling one to determine at a very early stage post-transplantation a likelihood of rejection because the primary graft rejection generally occurs in the biliary tree. |           |  |

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

|    |                          |    |  |    |                          |
|----|--------------------------|----|--|----|--------------------------|
| AM | Armenia                  | GB | United Kingdom                           | MW | Malawi                   |
| AT | Austria                  | GE | Georgia                                  | MX | Mexico                   |
| AU | Australia                | GN | Guinea                                   | NE | Niger                    |
| BB | Barbados                 | GR | Greece                                   | NL | Netherlands              |
| BE | Belgium                  | HU | Hungary                                  | NO | Norway                   |
| BF | Burkina Faso             | IE | Ireland                                  | NZ | New Zealand              |
| BG | Bulgaria                 | IT | Italy                                    | PL | Poland                   |
| BJ | Benin                    | JP | Japan                                    | PT | Portugal                 |
| BR | Brazil                   | KE | Kenya                                    | RO | Romania                  |
| BY | Belarus                  | KG | Kyrgyzstan                               | RU | Russian Federation       |
| CA | Canada                   | KP | Democratic People's Republic<br>of Korea | SD | Sudan                    |
| CF | Central African Republic | KR | Republic of Korea                        | SE | Sweden                   |
| CG | Congo                    | KZ | Kazakhstan                               | SG | Singapore                |
| CH | Switzerland              | LI | Liechtenstein                            | SI | Slovenia                 |
| CI | Côte d'Ivoire            | LK | Sri Lanka                                | SK | Slovakia                 |
| CM | Cameroon                 | LR | Liberia                                  | SN | Senegal                  |
| CN | China                    | LT | Lithuania                                | SZ | Swaziland                |
| CS | Czechoslovakia           | LU | Luxembourg                               | TD | Chad                     |
| CZ | Czech Republic           | LV | Latvia                                   | TC | Togo                     |
| DE | Germany                  | MC | Monaco                                   | TJ | Tajikistan               |
| DK | Denmark                  | MD | Republic of Moldova                      | TT | Trinidad and Tobago      |
| EE | Estonia                  | MG | Madagascar                               | UA | Ukraine                  |
| ES | Spain                    | ML | Mali                                     | UG | Uganda                   |
| FI | Finland                  | MN | Mongolia                                 | US | United States of America |
| FR | France                   | MR | Mauritania                               | UZ | Uzbekistan               |
| GA | Gabon                    |    |  | VN | Viet Nam                 |

Method of determining the hepatic status of an individual,  
including a liver transplant recipient

Technical Field

5        This invention relates to a method of determining the hepatic status of a subject, including a liver transplant recipient and, thereby, deciding on appropriate therapy or corrective action, if required, dependent on said hepatic status.

Background Art

10       The ability to differentiate between the various types of hepatic injury is of great significance in the treatment of both transplant patients and also individuals who suffer from other hepatic diseases which may affect the biliary system.

15       Glutathione S-transferases (GSTs) comprise a multigene family of proteins consisting mainly of alpha ( $\alpha$ GST), mu ( $\mu$ GST), pi ( $\pi$ GST) and theta-class ( $\theta$ GST) isoforms as defined by isoelectric point and are responsible for the detoxification of a range of xenobiotics, mainly *via* conjugation to glutathione (Beckett, G.J and Hayes, J.D., Advances in Clinical Chemistry (1993); 30, 281-380). Generally, the proteins are dimeric in nature consisting of two 25-27kDa subunits and may exist in  
20       homodimeric or heterodimeric forms. Pi Glutathione S-transferase ( $\pi$ GST) is a homodimer, and is located in the cytoplasm of bile duct epithelial cells within the liver (Beckett G.J. and Hayes, J.D., (1993) *supra*).  $\alpha$ GST is known to be present in hepatocytes within the liver and exists in both homodimeric and heterodimeric states (Campbell,  
25       J.A.H., *et. al.*, Cancer (Philadelphia) (1991) 67, 1608-1613; Howie, A.F., *et. al.*, Clin. Chem. Acta., (1988) 177, 65-76). This heterogenous GST distribution of  $\alpha$  and  $\pi$ GST suggests that the different isoenzymes have unique *in vivo* functions in different hepatic regions (Campbell, J.A.H., *et. al.*, (1991) *supra*).

EP-A 0 640 145 discloses a method which assists in the early diagnosis of rejection in a liver transplant recipient and which comprises measuring an increase in plasma or serum  $\alpha$ GST from the recipient in the absence of or preceding any change in plasma or serum transaminase. Thus, it has been conclusively demonstrated that  
5 measurement of the plasma  $\alpha$ GST level facilitates monitoring of the post-transplant hepatic status by acting as an extremely sensitive, although not totally specific marker of graft rejection.

It is notable that  $\pi$ GST has received no attention as a potential  
10 marker of graft rejection, a fact possibly due to the low levels of enzyme present in the biliary epithelial cells of the liver. There is some evidence, however, that  $\alpha$  and  $\pi$ GST are present in bile from both normal individuals and people suffering from specific cancers (e.g., cholangiocarcinoma) as measured by radio-immunoassay (Howie,  
15 A.F., *et al.*, Clin. Chem. Acta. (1989) 184, 269-278). Additionally, some authors have referenced the fact that measurement of serum and plasma  $\pi$ GST levels may facilitate diagnosis of malignant tumours since  $\pi$ GST appears to be specifically expressed in malignant tissue (Niitsu,  
20 Y., *et al.*, Cancer (1989) 63, 317-323; Howie, A.F., *et al.*, Clin. Chem. (1990) 36(3), 453-456. and Hida, T., *et al.*, Cancer (1994) 73(5), 1377-1382. None of the aforementioned authors allude to the fact that  $\pi$ GST may have a role in the prediction of transplanted liver rejection or other liver/biliary disorders.

Since it is known that primary graft rejection generally occurs in  
25 the biliary tree within the liver (Ascher, N., (1993) In 'Immunology of liver transplantation' Neuberger, J. and Adams, D. (eds)), it would appear that specific measurement of biliary or plasma  $\pi$ GST levels may allow diagnosis of early rejection or facilitate discrimination between post-transplant hepatocellular or biliary damage. The importance of  
30 distinguishing between non-specific hepatic injury and graft rejection cannot be overstated since the treatment for each condition is entirely different. Furthermore, initiation of the incorrect treatment could be extremely deleterious to the health of an individual already severely ill. For example, if graft injury occurs due to viral infection (e.g.,

Hepatitis C re-infection or cytomegalovirus (CMV), it is necessary to carefully monitor the levels of anti-rejection immunosuppression treatment since excess immunosuppressive agents (e.g., cyclosporin A or FK506) would significantly impair the ability to fight viral infection.  
5 Conversely, failure to recognise genuine rejection from non-specific graft injury could lead to delay in augmentation of immunosuppressive therapy and ultimately lead to graft removal.

Accordingly, there is a need for methods of determining the hepatic status of an individual in various disease states or abnormal  
10 conditions of the liver.

#### Disclosure of Invention

The invention provides a method of determining the hepatic status of a subject, which method comprises measuring the level of the pi glutathione S-transferase ( $\pi$ GST) isoform in a sample of a biological  
15 fluid from said subject by an immunoassay specific for the  $\pi$ GST isoform, comparing the level of  $\pi$ GST measured with the normal range of  $\pi$ GST in said biological fluid and, when an increase in  $\pi$ GST level relative to said normal range is detected, determining the hepatic status of the subject based on the level of  $\pi$ GST in said biological fluid.

20 By providing a further method for determining hepatic status based on a marker specific to a particular hepatic site greatly facilitates the treatment of patients with various disease states and other abnormal conditions of the liver as hereinafter described in greater detail.

The subject is suitably a liver transplant recipient and the hepatic  
25 status is determined post-transplantation.

The invention has particular application in the case of liver transplantation because it enables one to determine at a very early stage post-transplantation a likelihood of rejection because the primary graft rejection generally occurs in the biliary tree within the liver as stated  
30 above. Accordingly, even earlier detection of liver transplant rejection

is possible with the method according to the invention relative to the method described and claimed in EP-A 0 640 145.

Preferably, the recipient is a human.

5 The immunoassay is preferably an enzyme immunoassay, more especially a sandwich enzyme immunoassay.

The method according to the invention can be used to measure  $\pi$ GST in a range of media, but especially in bile, plasma and serum.

10 By biological fluid herein is meant for example body fluids such as bile, plasma, serum and urine as well as tissue support media and perfusates. The biological fluids herein are also referred to generally as matrices.

The method according to the invention facilitates for the first time detection of the  $\pi$ GST isoenzyme level in bile.

15 When the biological fluid is bile, the normal  $\pi$ GST level is less than 15  $\mu$ g/L.

When the biological fluid is plasma, the normal  $\pi$ GST level is less than 100  $\mu$ g/L.

20 As demonstrated hereinbelow care should be taken when the method is carried out on plasma that the plasma is collected and stored prior to the determination in the presence of an anti-coagulant under conditions which permit substantially no haemolysis to occur during said storage period.

25 We have found that use of fluoro-oxylate tubes results in a high degree of haemolysis releasing  $\pi$ GST from erythrocytes which gives falsely elevated levels of  $\pi$ GST. Other GST isoenzymes are either not found in the blood or are present at extremely low levels. For example,  $\mu$ GST is present in leucocytes. However, it is not clear from

the literature as to whether it is present in erythrocytes. In any event,  $\mu$ GST is only present in 50% of the population.  $\theta$ GST expresses a similar inter-individual variability as does  $\mu$ GST and if present in blood is present at extremely low levels.  $\alpha$ GST is not present in blood to any great extent.

In a preferred embodiment, the sample is diluted with a diluent which contains an effective amount of a protein which optimises antibody-antigen reactions.

We have found that if the diluent includes Tween 20 conventionally used as a standard reagent in such immunometric methods that incorrect  $\pi$ GST concentrations are detected. We have found that if one uses an effective amount of a protein which optimises antibody-antigen reactions, one can achieve a linear titration as shown in Example 6.

Suitably, the protein is a serum albumin such as bovine serum albumin or human serum albumin.

The immunoassay method according to the invention can be completed within 2.5 hours as hereinafter described in Examples. This is considerably faster than any commercially available assay for the quantitation of  $\pi$ GST.

The invention thus provides in one embodiment an immunoassay capable of being completed in under 2.5 hours which is based on the sequential addition of sample, antibody-enzyme conjugate and substrate to microtitre wells or other surface coated with monoclonal anti- $\pi$ GST IgG. The resultant colour intensity is proportional to the amount of  $\pi$ GST present in the sample and the assay range is 0-100  $\mu$ g/L. The assay range is readily extended by increased sample dilution.

According to another embodiment of the invention, additionally the level of the alpha glutathione S-transferase ( $\alpha$ GST) isoform is measured in a sample of a biological fluid from said subject so as to

facilitate differentiation between graft rejection and non-specific hepatocellular damage in said subject.

The invention also provides a test kit or pack containing one or more components for carrying out a method as hereinabove defined.

## 5 Brief Description of Drawings

In the accompanying drawings:

Fig. 1 is a schematic diagram of the sandwich enzyme immunoassay of Example 1;

10 Fig. 2 is a plot of absorbance at 450/630 nm *versus* log  $\pi$ GST concentration ( $\mu\text{g/L}$ ) according to the enzyme immunoassay for human  $\pi$ GST described in Example 1;

Fig. 3 is an SDS-PAGE analysis of human  $\mu$ ,  $\alpha$  and  $\pi$ GST;

Fig. 4 is an immunoblot analysis of human  $\pi$ GST;

15 Fig. 5 is a plot of  $\alpha$ GST and  $\pi$ GST (ng/ml) in bile *versus* time after reperfusion (hours) for a number of patients;

Fig. 6 is a plot of  $\pi$ GST concentration *versus* time (days) for a number of patients; and

Fig. 7 is a plot of AST/ALT (U/L) and  $\alpha$ GST and  $\pi$ GST (ng/ml) *versus* days after transplantation for a single patient.

## 20 Modes for Carrying Out the Invention

The invention will be further illustrated by the following Examples.



### Preparatory Example A

#### Purification of human $\pi$ GST

$\pi$ GST was purified from human placenta by affinity chromatography. Precise details of the purification procedure are as follows:

- a. 325g of human placenta was homogenised for 2 minutes in homogenisation buffer, at a ratio of one part placenta to three parts buffer, using a Waring (Waring is a Trade Mark) blender. The homogenisation buffer had the following composition:
  - 20mM Tris-HCl
  - 250mM Sucrose
  - 5mM EDTA                      pH 7.8
  - 2 $\mu$ g/ml Leupeptin
  - 2 $\mu$ g/ml Pepstatin.
- b. The placenta homogenate was centrifuged at 10000g for 60 minutes.
- c. The supernatant was then loaded on a Glutathione (GSH)-Sepharose Affinity column previously equilibrated in 20mM Tris-HCl with 200mM NaCl, pH 7.8. Equilibration buffer was reapplied to elute unbound protein. Finally 50mM Tris-HCl pH 9.5 containing 5mM GSH was used to elute bound GST from the affinity column.
- d. The eluted material was then dialysed against 0.1M PBS.

### Preparatory Example B

#### Antibody Production and Purification:

- Purified human  $\pi$ GST was injected into New Zealand White rabbits subcutaneously (s.c.) according to the time schedule given

below and serum evaluated for anti- $\pi$ GST reactivity. Once the IgG [anti-human  $\pi$ GST] titre was sufficient as determined by semi-quantitative dot blot analysis, the animals were exsanguinated and serum collected. Total IgG was purified from rabbit serum by Protein  
5 A affinity chromatography and was used for conjugation to horseradish peroxidase (HRP). Monoclonal IgG [anti-human  $\pi$ GST] as ascites, was obtained from The University Hospital, Nijmegen, The Netherlands and was not purified further prior to use.

Immunisation Schedule (general):

10 Day 1: A test bleed of 5ml of preserum was taken from the ear of the rabbit. 0.5ml of human  $\pi$ GST antigen (100 $\mu$ g) was mixed with an equal volume of Freund's Complete Adjuvant. The mixture of antigen and adjuvant was homogenised to ensure a good emulsion. This  
15 mixture was then injected subcutaneously into multiple sites on the back of the rabbit which had previously been shaved.

Day 28: A test bleed of 5ml of serum was taken from the ear of the rabbit. 0.5ml antigen (100 $\mu$ g) was mixed with an equal volume of Freund's Incomplete Adjuvant. The antigen/adjuvant mixture was  
20 homogenised to ensure a good emulsion. This mixture was then injected subcutaneously into multiple sites on the back of the rabbit.

Day 42: A test bleed of 10ml of blood was taken from the rabbit's ear.

Day 56: A second boost was given to the rabbit as described on Day 28.

25 Day 70: A test bleed of 10ml of blood was taken from the ear of the rabbit. When the titre was sufficiently high, the rabbit was sacrificed and as much blood as possible collected.

Preparatory Example CImmunoblotting:

5 All polyclonal and monoclonal IgGs for use in the followings  
Examples were checked for  $\pi$ GST reactivity and potential cross-  
reactivity against human  $\alpha$  and  $\mu$ GST respectively, via the following  
immunoblot combinations:-

- (a) Rabbit IgG [anti-human  $\pi$ GST] was used to probe  
nitrocellulose membranes containing immobilised  
human  $\alpha$ ,  $\pi$  and  $\mu$ GST.
- 10 (b) Murine IgG [anti-human  $\pi$ GST] was used to probe  
nitrocellulose membranes containing immobilised  
human  $\alpha$ ,  $\pi$  and  $\mu$ GST.

The method used for immunoblot detection was as follows:

- 15 1. Human  $\alpha$ ,  $\pi$  and  $\mu$ GST (0.5 $\mu$ g/track) were electrophoresed  
on 15% SDS-PAGE with molecular weight markers also included.
2. After electrophoresis, the polyacrylamide gel was cut and  
one half stained for protein while the remainder was used for  
electrophoretic transfer onto nitrocellulose.
- 20 3. After electrophoretic transfer, the nitrocellulose  
membranes were blocked for 1 hour with 5%(w/v) Marvel (Marvel is  
a Trade Mark) in phosphate buffered saline containing 0.05%(w/v)  
TWEEN-20 (PBST)- blocking buffer.
4. The following solutions were then prepared:
- 25 (i) Rabbit IgG [anti-human  $\pi$ GST] in 1%(w/v) Marvel  
in PBST

- (ii) Murine IgG [anti-human  $\pi$ GST] in 1%(w/v) Marvel in PBST

and added to the membranes once blocking buffer was decanted.

5 Incubation with antibody solutions was allowed to proceed for one hour.

5. The nitrocellulose membranes were then washed in PBST (2x for 5 min each).

10 6. Anti rabbit IgG-HRP conjugate was then prepared (1/1000 in 1% (w/v) Marvel in PBST and added to 4(i) above. Anti murine IgG-HRP conjugate was also prepared (1/1000) and added to 4(ii) above.

7. After one hour incubation with anti-species conjugates, the reagents were discarded and the membranes washed as in 5 above.

15 8. Diaminobenzidine substrate was then prepared and added to the membrane.

A positive reaction was indicated by a brown precipitate on the nitrocellulose membrane.

#### Preparatory Example D

##### Anti $\pi$ GST IgG-horseradish peroxidase (HRP) conjugate synthesis:

20 Anti  $\pi$ GST IgG-HRP conjugates were synthesised using thioether conjugation methodology. (Duncan, R.J.S., *et al.*, (1983); Anal. Biochem. 132, 68-73) Reactive maleimide groups were introduced onto IgG molecules using SMCC (succinimidyl 4-(N-maleimidomethyl) cyclohexane 1-carboxylate) and masked sulphydryl groups were linked  
25 to HRP. After a demasking step to produce reactive sulphydryl groups, the maleimide-activated IgG and HRP-SH were mixed together and

allowed to react for 4.5 hours. The resultant IgG-HRP conjugate, formed by covalent thioether linkage, was brought to 50% (v/v) glycerol and stored at  $-20^{\circ}\text{C}$  for use in the EIA of Example 1.

### Example 1

#### 5                      Sandwich enzyme immunoassay

The format of the immunoassay for the quantitative detection of human  $\pi\text{GST}$  is a conventional sandwich format as depicted schematically in Fig. 1, and described below.

10                      a.     A Nunc Maxisorp (Nunc Maxisorp is a Trade Mark) microtitre plate was coated with murine monoclonal IgG [anti-human  $\pi\text{GST}$ ] (referred to in Preparatory Example B) immobilised *via* goat  $\text{F(ab)}_2$  fragments [anti-mouse IgG]. This method of antibody coating serves to orientate Mab binding sites and also improves assay sensitivity by minimising adherence - induced denaturation of the capture  
15                      antibody.

b.     Human  $\pi\text{GST}$ , purified from placenta as described in Preparatory Example A, was used as the assay calibrator.

20                      c.     IgG [anti-human  $\pi\text{GST}$ ]-HRP conjugates, in association with tetramethylbenzidine substrate (TMB), were used to facilitate detection of captured/immobilised  $\pi\text{GST}$ .

25                      d.     The enzyme reaction was stopped by the addition of 1N  $\text{H}_2\text{SO}_4$  and the absorbance measured at 450nm using 630nm as a reference wavelength. Colour intensity was proportional to  $\pi\text{GST}$  concentration and after generating a plot of  $A_{450/630\text{nm}}$  *versus* concentration ( $\mu\text{g/L}$ ), the concentration of unknown samples can be determined (see Fig. 2). Total assay time was less than 2.5 hours.

The total assay time was found to be 2 hours 15 minutes and assay conditions included microtitre plate shaking at fixed temperature

during the sample and conjugate incubation steps, respectively. The TMB substrate incubation required fixed temperature conditions only.

### Example 2

#### Collection of plasma for $\pi$ GST analysis

5           The effect of a variety of commonly used anti-coagulants (ethylenediaminetetraacetic acid (EDTA), lithium heparin, sodium citrate and fluoro-oxylate) and other plasma collection tubes (containing platelet inhibitors) on  $\pi$ GST levels in plasma, was examined. Samples were collected by venipuncture into tubes  
10           containing the particular anticoagulant. Plasma was separated by centrifugation (6,000g for 10 min), and residual platelets were removed by an additional centrifugation step (10,000g for 10 min). The supernatant was removed, and samples were assayed using the protocol described in Example 1.

15           Plasma was collected from a number of individuals into a series of plasma collection tubes. Each sample was handled as described above, and the release of  $\pi$ GST into plasma was monitored over a 24 hour period. Table 1 below shows the  $\pi$ GST levels in plasma from the same individual collected into four different plasma collection tubes.  
20           assayed at T0 and T24. The results show that there is no significant difference in  $\pi$ GST concentrations in plasma collected in the presence of any of the above anti-coagulants. There does not appear to be a significant increase in  $\pi$ GST concentrations (caused by release of  $\pi$ GST from erythrocytes or platelets) when the unseparated plasma is stored  
25           for up to 24 hours. The exception is the fluoro-oxylate tubes where a large degree of haemolysis occurred, releasing  $\pi$ GST from erythrocytes, and giving falsely elevated levels of the protein as shown in Table 1.

Table 1

Comparison of the influence of anti-coagulants in plasma collection tubes for  $\pi$ GST analysis in plasma. Values are given in  $\mu\text{g/L}$ .

| Patient | EDTA  |       | Li/Hep |       | Na citrate |       | Fl oxylate |      |
|---------|-------|-------|--------|-------|------------|-------|------------|------|
|         | T0    | T24   | T0     | T24   | T0         | T24   | T0         | T24  |
| 1       | 171.9 | 220.2 | 199.4  | 194.7 | 167.5      | 220.3 | 170.0      | >500 |
| 2       | 65.6  | 70.5  | 62.8   | 88.4  | 53.2       | 65.4  | 55.4       | >500 |
| 3       | 59.6  | 78.6  | 65.4   | 88.6  | 53.7       | 68.4  | 59.9       | >500 |
| 4       | 81.7  | 92.8  | 90.2   | 119.5 | 74.9       | 122.1 | 81.2       | >500 |
| 5       | 46.7  | 67.5  | 44.6   | 73.3  | 28.9       | 68.6  | 60.9       | >500 |

5 It can be seen from Table 1 that there is no significant difference between the concentration of  $\pi$ GST in plasma collected in the presence of any of the above anti-coagulants.

### Example 3

#### Assessment of purity of immunoassay reagents

10 Fig. 3 illustrates the purity of human  $\pi$ GST obtained by the procedure of Preparatory Example A prior to immunisation into rabbits and confirms the absence of any other human derived proteins which might otherwise contribute to reduced assay specificity.  
In Fig. 3:

15 Lane 1 =  $\mu$ GST  
Lane 2 =  $\alpha$ GST  
Lane 3 =  $\pi$ GST  
Lane 4 = molecular weight markers.

20 Immunoblot analysis of the monoclonal antibody reactivity revealed that the IgG[anti-human  $\pi$ GST] was highly specific for human  $\pi$ GST and did not exhibit any significant cross-reactivity with human  $\alpha$  or  $\mu$ GST. The results are shown in Fig. 4.

Lane 1 =  $\pi$ GST

Lane 2 = molecular weight markers.

A finding supported by the lack of  $\alpha$  and  $\mu$ GST reactivity in the human  $\pi$ GST-specific enzyme immunoassay as shown in Table 2.

5

Table 2

Evaluation of human  $\alpha$ GST and  $\mu$ GST reactivity in the enzyme immunoassay for the detection of human  $\pi$ GST.

| GST<br>Conc. | A <sub>450/630nm</sub> |              |           |
|--------------|------------------------|--------------|-----------|
|              | $\pi$ GST              | $\alpha$ GST | $\mu$ GST |
| 0.00         | 0.022                  | 0.022        | 0.022     |
| 3.12         | 0.087                  | 0.024        | 0.016     |
| 12.5         | 0.299                  | 0.020        | 0.022     |
| 25.0         | 0.688                  | 0.036        | 0.055     |
| 50.0         | 1.204                  | 0.018        | 0.034     |
| 100          | 1.735                  | 0.040        | 0.043     |

It is clear from Table 2 that no cross-reactivity is evident for either  $\alpha$ GST or  $\mu$ GST in this assay.

10

The significance of this fact is of utmost importance since it implies that the enzyme immunoassay for human  $\pi$ GST quantitation is specific for the detection of human  $\pi$ GST. Thus, any human  $\pi$ GST present in samples can be specifically detected without cross-contamination from other GSTs.

15

Example 4

$\pi$ GST quantitation in bile

A number of bile samples from patients with specific liver/biliary damage were tested in the assay for human  $\pi$ GST. Patients with hepatocellular carcinoma (HCC) and primary biliary cirrhosis (PBC) were found to contain very high levels of  $\pi$ GST. A patient with

20



bile duct stones (BDS) was also found to have increased  $\pi$ GST levels, but not as elevated as those for HCC and PBC as shown in Table 3.

Table 3

5  $\pi$ GST concentrations in bile from patients with hepatocellular carcinoma (HCC), primary biliary cirrhosis (PBC) and bile duct stone (BDS).

| Condition | [ $\pi$ GST] ( $\mu$ g/L) |
|-----------|---------------------------|
| HCC       | 5357                      |
| PBC       | 2432                      |
| BDS       | 506.6                     |

All of these samples show significantly elevated  $\pi$ GST levels.

Example 5

Clinical utility of  $\pi$ GST quantitation

10 Serial bile and plasma samples were collected from patients following liver transplant operations and assayed for both  $\pi$  and  $\alpha$ GST respectively. The patients exhibited a range of post-operative conditions, from uneventful recovery, to acute rejection and Hepatitis C re-infection, which are normally associated with transplantation.

15 A number of significant trends were observed when the concentrations of both  $\pi$  and  $\alpha$ GST in bile were monitored simultaneously.  $\alpha$ GST was measured according to the procedure described in EP-A 0 640 145. During an uneventful recovery,  $\pi$ GST could be detected as soon as 2 hours post transplantation, and levels remained low (*i.e.* below 50  $\mu$ g/L).  $\alpha$ GST levels were initially high due to reperfusion injury, but returned to baseline levels within 2 days as shown in Fig. 5. This figure shows the typical course of  $\alpha$  and  $\pi$ GST during and after human liver transplantation.  $\pi$ GST levels remain low. Complications associated with liver transplantation could

20

also be identified. One of the major risks is acute rejection, or the even more serious, steroid-resistant rejection. We have found that in these particular cases, there was a significant increase in  $\pi$ GST levels sustained over a period of days as shown in Fig. 6. This figure shows  $\pi$ GST levels during episodes of steroid-resistant rejection (SR) and acute rejection (AR). Levels are elevated and remain high over a period of at least 20 days.  $\alpha$ GST levels were also high, but they returned to baseline levels within a period of 5-8 days.

The possibility of infection or re-infection is another serious risk involved in transplantation. We have seen that in episodes of HCV re-infection, very high levels of  $\alpha$ GST were observed over a sustained period of time (*i.e.* at least 25 days). However,  $\pi$ GST levels remained near normal as shown in Fig. 7. This contrasts sharply with the previous cases of acute rejection, where the reverse was true. Thus, by the simultaneous quantitation of  $\alpha$  and  $\pi$ GST, it was possible to successfully differentiate between acute rejection and HCV re-infection, something which has previously proved to be very difficult, and has posed a therapeutic dilemma to transplant surgeons.

### Example 6

#### Linearity of dilution of bile samples

A number of bile samples were obtained from patients with specific liver/biliary damage (hepatocellular carcinoma and primary biliary cirrhosis), as well as samples from donor bile, and post liver transplantation. These samples were assayed for human  $\pi$ GST according to the protocol of Example 1.

A number of standard diluents were used as sample diluents for the titration of the bile samples. These diluents are routinely used in many assay systems, with Tween-20 being the most commonly used detergent. We have found however that the presence of this particular detergent in the sample diluent caused erroneous results. Falsely high concentrations of  $\pi$ GST were observed in samples diluted in Tween-20-

containing diluents, caused by insufficient titration as shown in Table 4. In the absence of Tween-20, linear titration was observed. Therefore, a critical factor of this assay is the absence of Tween-20 (a standard reagent) in the sample diluent, as falsely elevated levels of  $\pi$ GST would be observed if it were used.

Table 4

Comparison of the titration of a PBC bile sample in diluents with and without Tween-20. Values are given in  $\mu\text{g/L}$ .

| Dilution | - Tween-20 | + Tween-20 |
|----------|------------|------------|
| 1/50     | 2344       | 3074       |
| 1/100    | 2374       | 5187       |
| 1/200    | 2410       | 7059       |

Linear titration was observed only in the diluent without Tween-20.

Example 7

Comparison of Polyclonal and Monoclonal Antibodies as coating antibodies

Polyclonal and monoclonal anti-human  $\pi$ GST IgG were immobilised onto Nunc Maxisorp plates either directly or *via* a linker ( $\text{F(ab)}_2$  fragments of goat anti-species IgG). Standards of known concentrations of human  $\pi$ GST were then run as described in Example 1 and the absorbances at similar immobilised IgG concentrations compared.

Both polyclonal and monoclonal antibodies were coated onto the solid phase for use as a capture antibody. Direct coating of the both the polyclonal and the monoclonal antibodies resulted in very low absorbance readings for the standard curve (see Table 5). When immobilisation was achieved via a linker antibody (goat anti-

mouse/anti-rabbit IgG). a significant increase in O.D. values was obtained for the monoclonal antibody. However, no such increase was observed for the polyclonal antibody.

Table 5

5 Comparison of coating methods for the detection of human  $\pi$ GST.

| [ $\pi$ GST] | DIRECT COATING |            | COATING <i>via</i> LINKER ANTIBODY |                |
|--------------|----------------|------------|------------------------------------|----------------|
|              | Polyclonal     | Monoclonal | Polyclonal                         | Monoclonal Ab. |
| 100          | 0.625          | 0.421      | 0.352                              | 1.557          |
| 0            | 0.044          | 0.038      | 0.235                              | 0.095          |

Direct coating of the antibody onto the solid phase (2  $\mu$ g/mL) was compared to coating *via* a linker antibody (goat anti species, at 2  $\mu$ g/ml). with the anti  $\pi$ GST antibody at a fixed concentration.

Claims:-

1. A method of determining the hepatic status of a subject, which method comprises measuring the level of the pi glutathione S-transferase ( $\pi$ GST) isoform in a sample of a biological fluid from said  
5 subject by an immunoassay specific for the  $\pi$ GST isoform, comparing the level of  $\pi$ GST measured with the normal range of  $\pi$ GST in said biological fluid and, when an increase in  $\pi$ GST level relative to said normal range is detected, determining the hepatic status of the subject based on the level of  $\pi$ GST in said biological fluid.
- 10 2. A method according to Claim 1, wherein the subject is a liver transplant recipient and the hepatic status is determined post-transplantation.
3. A method according to Claim 1 or 2, wherein the subject is a human.
- 15 4. A method according to any preceding claim, wherein the immunoassay is an enzyme immunoassay.
5. A method according to any preceding claim, wherein the biological fluid is bile and the normal  $\pi$ GST level is less than 15  $\mu$ g/L.
- 20 6. A method according to Claim 4, wherein the biological fluid is plasma and the normal  $\pi$ GST level is less than 100  $\mu$ g/L.
7. A method according to Claim 6, wherein the plasma is collected and stored prior to the determination in the presence of an anti-coagulant under conditions which permit substantially no haemolysis to occur during said storage period.
- 25 8. A method according to any preceding claim, wherein the sample is diluted with a diluent which contains an effective amount of a protein which optimises antibody-antigen reactions.

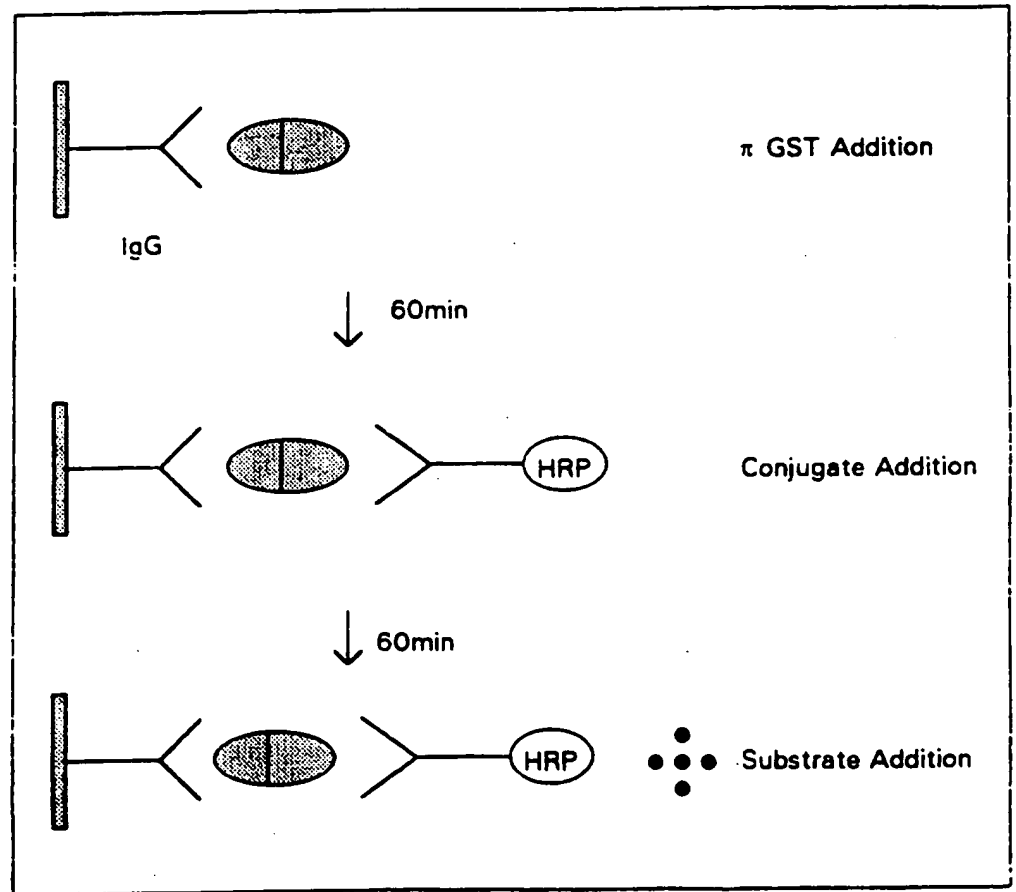
9. A method according to Claim 8, wherein the protein is a serum albumin.

10. A method according to any preceding claim, wherein the entire immunoassay is completed within 2.5 hours.

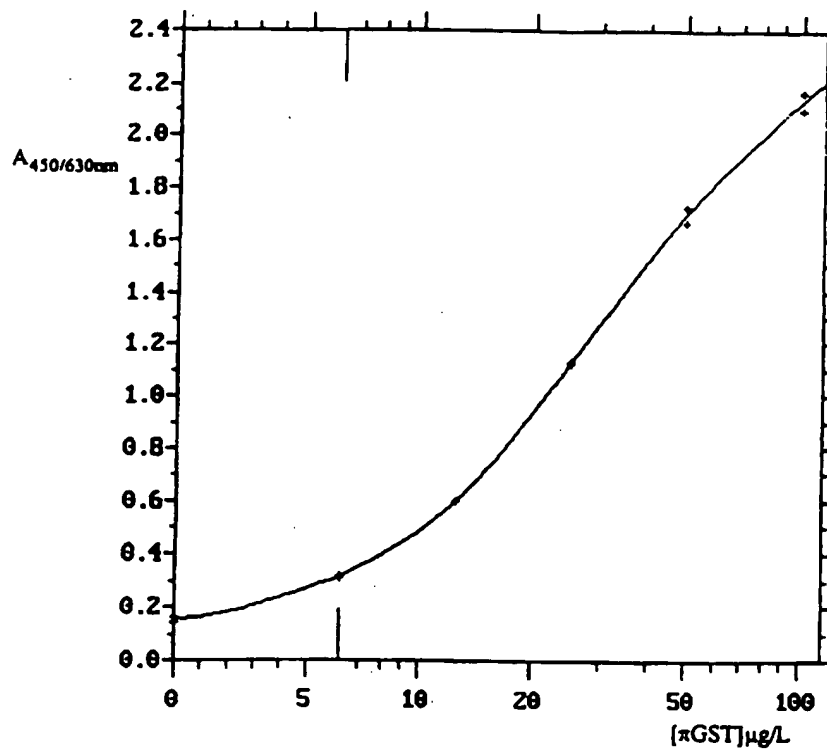
5 11. A method according to any one of Claims 2-10, wherein additionally the level of the alpha glutathione S-transferase ( $\alpha$ GST) isoform is measured in a sample of a biological fluid from said subject so as to facilitate differentiation between graft rejection and non-specific hepatocellular damage in said subject.

10 12. A test kit or pack containing one or more components for carrying out a method according to any one of Claims 1-11.

1/7

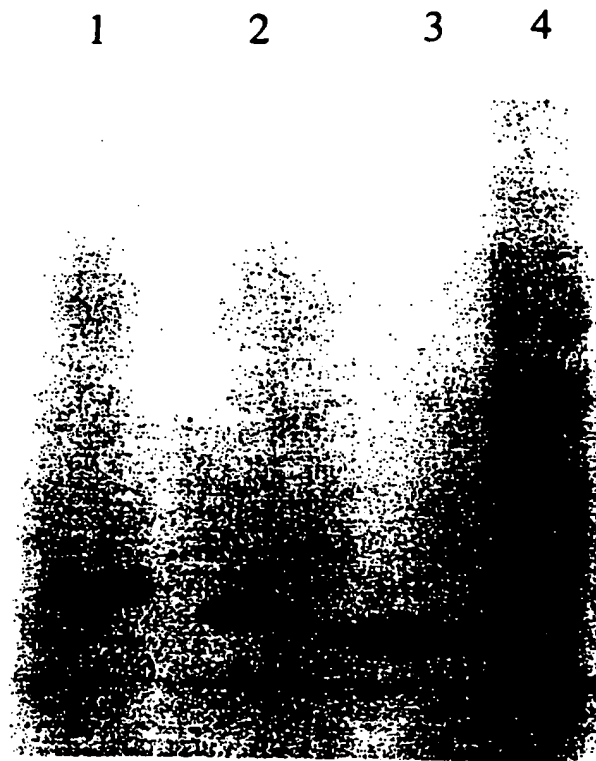
**Fig. 1**

2/7

**Fig. 2**



3/7



**Fig. 3**

4/7

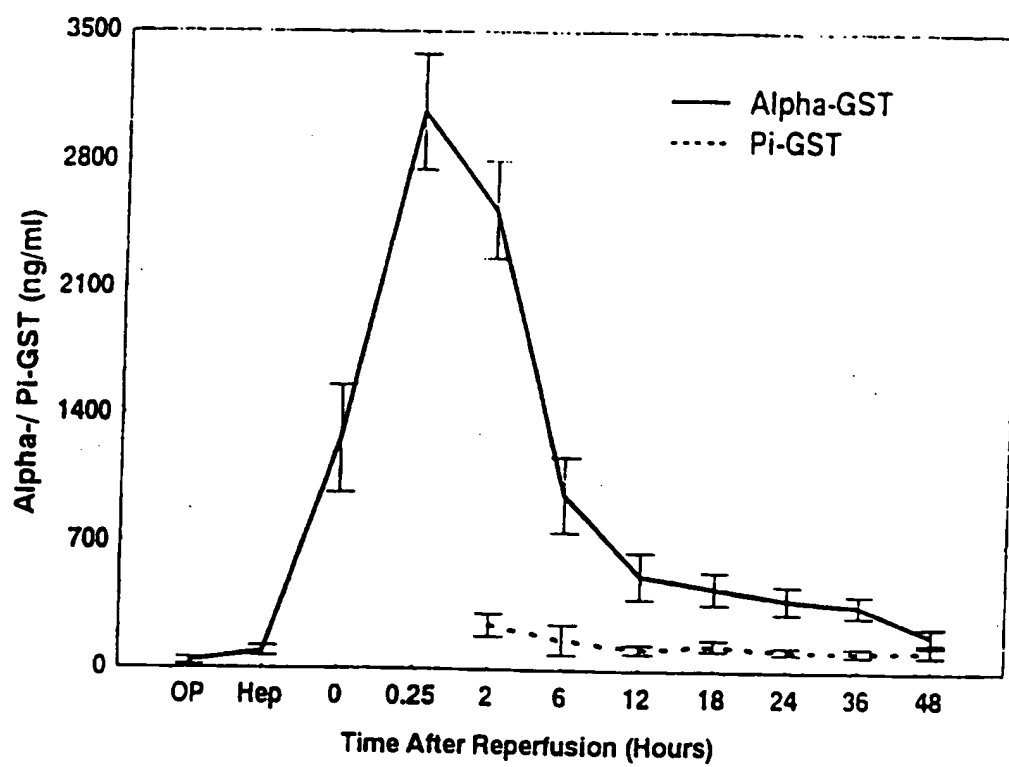
1

2

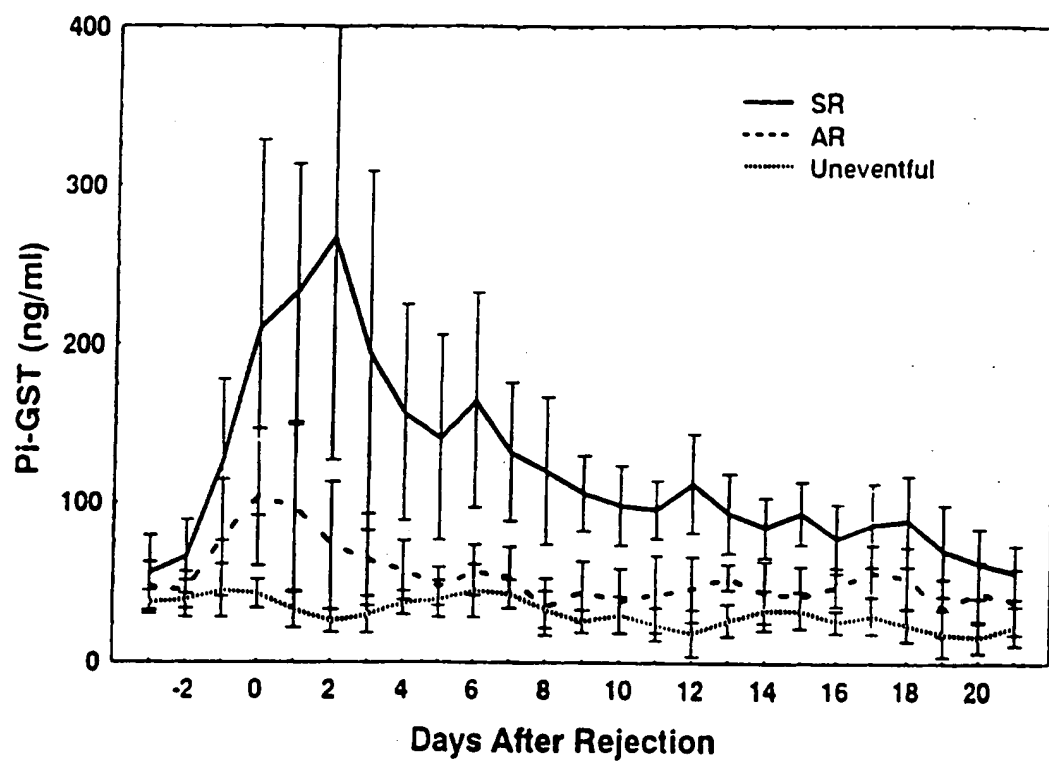


**Fig. 4**

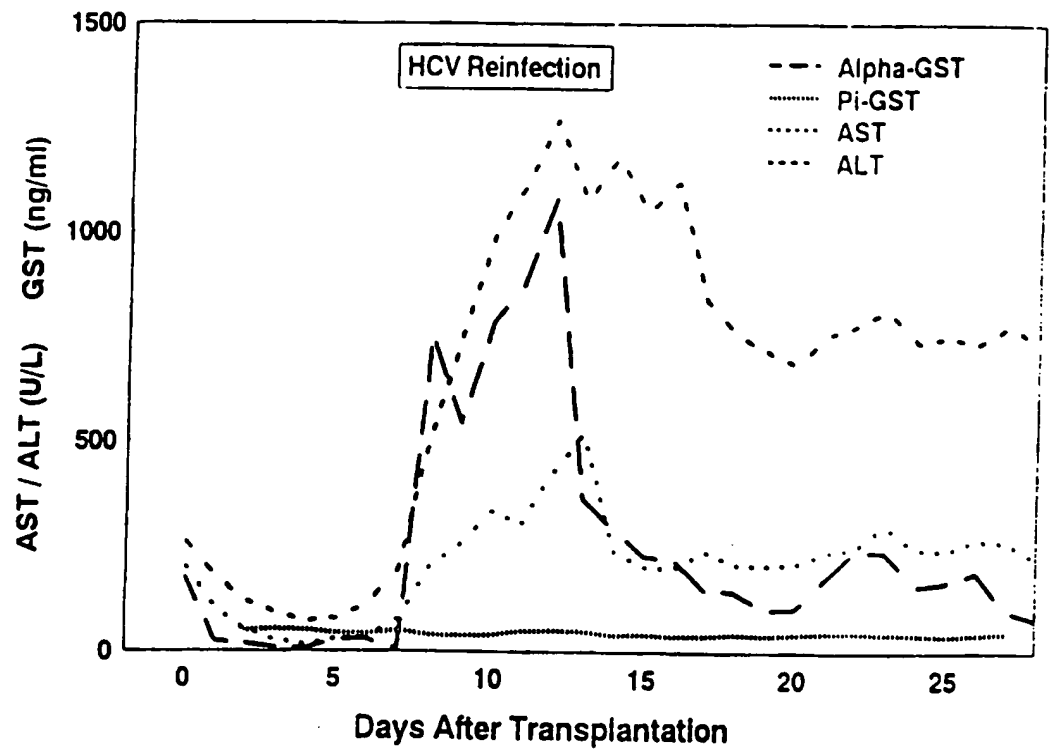
5/7

**Fig. 5**

6/7

**Fig. 6**

7/7

**Fig. 7**

## INTERNATIONAL SEARCH REPORT

International Application No

PL./IE 96/00003

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 G01N33/573

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages  | Relevant to claim No. |
|------------|---|-----------------------|
| Y          | WO,A,93 22452 (C. G. KILTY) 11 November 1993<br>see the whole document<br>& EP,A,0 640 145 (...)<br>cited in the application<br>--- | 1-12                  |
| Y          | WO,A,90 12088 (THE UNITED STATES OF AMERICA) 18 October 1990<br>see the whole document<br>---                                       | 1-12                  |
|            | -/--  |                       |

☒ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

- \*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- \*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- \*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- \*&\* document member of the same patent family

Date of the actual completion of the international search

1 October 1996

Date of mailing of the international search report

18.10.96

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Griffith, G

## INTERNATIONAL SEARCH REPORT

 International Application No  
 PC./IE 96/00003

| C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT |  |                       |
|---|--|-----------------------|
| Category *  | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
| Y   | CHEMICAL ABSTRACTS, vol. 109, no. 25,<br>19 December 1988<br>Columbus, Ohio, US;<br>abstract no. 225320,<br>Y. HIRATA.: "The development of<br>immunoradiometric assay of glutathione<br>S-transferase (GST)-pi and its application<br>to serodiagnosis in cancers of<br>gastrointestinal tract."<br>page 345; column 2;<br>XP002014847<br>see abstract<br>& SAPPORO IGAKU ZASSHI,<br>vol. 57, no. 3, 1988,<br>pages 277-290,<br>--- | 1-12                  |
| Y   | CHEMICAL ABSTRACTS, vol. 118, no. 9,<br>1 March 1993<br>Columbus, Ohio, US;<br>abstract no. 78246,<br>L.ZHU ET AL.: "Detection of glutathione<br>S-transferase in human hepatocellular<br>carcinoma and other chronic liver disease<br>tissue."<br>page 560; column 2;<br>XP002014848<br>see abstract<br>& SHANGHAI YIKE DAXUE XUEBAO,<br>vol. 19, no. 4, 1992,<br>pages 263-266,<br>---   | 1-12                  |
| A   | CHEMICAL ABSTRACTS, vol. 117, no. 23,<br>7 December 1992<br>Columbus, Ohio, US;<br>abstract no. 231211,<br>T. SATO.: "Immunohistochemical<br>localization of human glutathione<br>S-transferase isozymes in primary liver<br>tumors and intrahepatic tumor-like<br>lesions."<br>page 599; column 2;<br>XP002014849<br>see abstract<br>& KANZO,<br>vol. 33, no. 5, 1992,<br>pages 390-398,<br>---                                     | 1-12                  |
| A   | DATABASE WPI<br>Section Ch, Week 9211<br>Derwent Publications Ltd., London, GB;<br>Class B04, AN 92-083782<br>XP002014850<br>& JP,A,04 025 763 (MARUKO SEIYAKU KK) . 29<br>January 1992<br>see abstract<br>-----   | 1-12                  |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PL./IE 96/00003

| Patent document<br>cited in search report | Publication<br>date | Patent family<br>member(s)                      | Publication<br>date              |
|---|---------------------|---|----------------------------------|
| WO-A-9322452                              | 11-11-93            | EP-A- 0640145<br>JP-T- 7506428<br>US-A- 5217868 | 01-03-95<br>13-07-95<br>08-06-93 |
| WO-A-9012088                              | 18-10-90            | AU-A- 5435990                                   | 05-11-90                         |